

Synaptotagmin is endogenously phosphorylated by Ca^{2+} /calmodulin protein kinase II in synaptic vesicles

Maurizio Popoli

Laboratory of Neurochemistry, II School of Medicine, Via S. Pansini 5, 80131 Naples, Italy

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The cytoplasmic domain of synaptotagmin (a synaptic vesicle-specific protein) has a high degree of homology with the Ca^{2+} -phospholipid binding domain of protein kinase C. The Ca^{2+} -phospholipid binding activity of synaptotagmin has been implicated in the docking and fusion of synaptic vesicles with the presynaptic membrane during Ca^{2+} -induced exocytosis. The protein sequence contains potential phosphorylation sites for various protein kinases which could modulate its binding activity. At present there is no clear evidence that the protein is endogenously phosphorylated in intact vesicles. Here it is reported that phospho-synaptotagmin was immunoprecipitated from endogenously phosphorylated synaptic vesicles.

The conditions used indicate that synaptotagmin, as synapsin I, is phosphorylated by Ca^{2+} /calmodulin-dependent protein kinase II.

p65-synaptotagmin; Synaptic vesicle; Neurotransmitter release; Protein phosphorylation; Calcium/calmodulin kinase II

1. INTRODUCTION

Synaptotagmin is an integral membrane protein specifically localized on secretory vesicles of neurons (synaptic vesicles) and on some endocrine cells (chromaffin granules) [1–3]. An increasing body of evidence recently suggested that the protein may have a role as a docking–fusion protein in synaptic vesicle exocytosis [2–7]. Synaptotagmin is a highly conserved protein and its cytoplasmic portion has a high degree of homology with the Ca^{2+} - and phospholipid-binding domain of protein kinase C (PKC) [2]. The protein binds Ca^{2+} at physiological concentrations in a complex with acidic phospholipids and its binding properties may match the requirements of a Ca^{2+} receptor in synaptic vesicle exocytosis [6,9]. The protein has been also found associated in presynaptic membranes with ω -conotoxin-sensitive Ca^{2+} channels (mediating the Ca^{2+} influx involved in neurotransmitter release), and with the α -latrotoxin receptor (toxin binding to the receptor causes a massive release) [4,10,11]. As the synaptotagmin sequence contains potential phosphorylation sites for PKC, casein kinase II and Ca^{2+} /calmodulin dependent kinase II (CaMK II) [3], phosphorylation of the protein could be important for the regulation of its Ca^{2+} –phospholipid binding activity and for synaptotagmin–receptor interactions. Partial evidence that the protein is

phosphorylated has been presented. Detergent-solubilized synaptotagmin was reported to be endogenously phosphorylated by an unknown kinase [4]. Also the protein solubilized or present in a crude vesicle fraction was phosphorylated upon addition of pure PKC or CaMK II [12]. In a recent report the protein was endogenously phosphorylated in a detergent-solubilized immunocomplex by a kinase reported as casein kinase II [7]. There is at present no clear demonstration of synaptotagmin phosphorylation in intact vesicles. Here it is reported that this protein is endogenously phosphorylated in intact synaptic vesicles by CaMK II.

2. MATERIALS AND METHODS

2.1. Materials

Monoclonal antibody (MAb) for synaptotagmin was kindly donated by Dr. E. Floor (Lawrence, KA). Antiserum for synapsin I was kindly donated by Dr. F. Benfenati (Modena, Italy). Alkaline phosphatase-conjugated second antibodies and calmodulin were purchased from Sigma. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol) was from NEN. Protein A-Sepharose CL-4B was from Pharmacia.

2.2. Synaptic vesicle purification

Synaptic vesicles (SV) were purified from rat forebrain as previously reported [8].

2.3. SV protein phosphorylation

Endogenous protein phosphorylation of SV was carried out in buffer A: 25 mM HEPES, pH 7.4, 10 mM magnesium acetate, 0.1 mM DTT, 1 mM EGTA or (in alternative) 0.2 mM CaCl_2 , 5 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4–10 Ci/mmol), and (when present) 5–20 $\mu\text{g}/\text{ml}$ calmodulin. The vesicle protein concentration was 0.7 mg/ml. Vesicles were incubated 1 min at 30°C. The reaction was terminated by adding half the incubation volume of electrophoresis sample buffer 3 \times concentrated, followed by boiling for 2 min. Phosphorylated proteins were analyzed by denaturing electrophoresis [5]. The gels were dried and autoradiographed. Immunoblotting was carried out as previously described [5].

Correspondence address: M. Popoli, c/o CNR Center of Cytopharmacology, Dipartimento di Farmacologia Medica, Università di Milano, Via Vanvitelli 32, 20129 Milano, Italy.

Abbreviations: PKC, protein kinase C; CaMK II, calcium/calmodulin-dependent protein kinase II, MAb, monoclonal antibody; SV, synaptic vesicles

2.4. Immunoprecipitation

A modification of a published method was used [13]. Vesicles phosphorylated for 1 min were brought to 0.5% SDS, boiled 3 min and diluted 5× with buffer B (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1% Triton X-100, pH 7.4). Synaptotagmin-MAb was added (60 μ l/100 μ g of SV protein) and the mixture incubated overnight at 4°C with slow end-over-end rotation. Control samples were incubated with synapsin I-antiserum (1 μ l/100 μ g of SV protein), or purified MAb OKT8 (2 μ l/100 μ g of SV protein). At the end of incubation protein A-Sepharose was added (1:1 slurry, 40 μ l/100 μ g of SV protein), and the mixture incubated for 2.5 h at 4°C. Protein A-Sepharose was pelleted, washed 2× with buffer B containing 0.6 M NaCl, 3× with buffer B, resuspended in 60 μ l of SDS sample buffer and boiled. The supernatant was analyzed by SDS electrophoresis and autoradiography.

For immunoprecipitation of the synaptotagmin cytoplasmic portion, 100 μ g of phosphorylated vesicles were incubated 30 min at 37°C with various trypsin concentrations. Incubation was stopped adding a 10× the amount of soy bean trypsin inhibitor and bringing the samples to 0.2 mM PMSF, followed by centrifugation for 1 h at 145,000 × g at 4°C. The supernatant was diluted 5× with buffer B and subsequently treated as above.

3. RESULTS

When synaptic vesicles were incubated in a standard phosphorylation buffer (in the presence or absence of Ca^{2+}) little or no endogenous phosphorylation of vesicle proteins was seen, except for one band with apparent M_r 50 K (Fig. 1). When calmodulin (20 μ g/ml) was added to the incubation mixture it markedly stimulated the phosphorylation of a number of proteins. The most

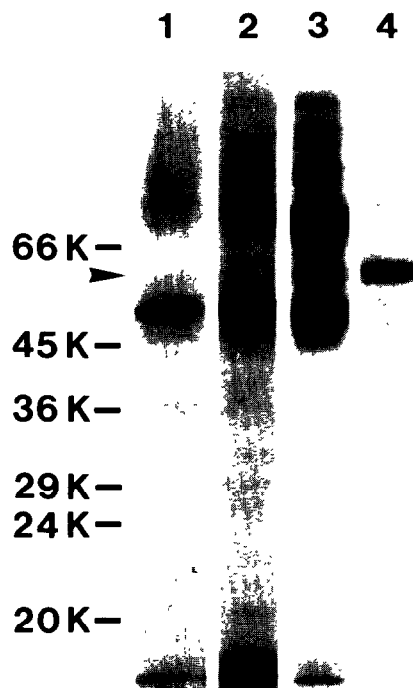


Fig. 1. Endogenous phosphorylation of synaptic vesicle protein. 50 μ g of vesicle proteins were phosphorylated for 1 min in buffer A in the presence of: 1, 1 mM EGTA; 2, 0.2 mM CaCl_2 ; 3, 0.2 mM CaCl_2 + 20 μ g/ml calmodulin. Lane 4, immunoblot of the same amount of vesicle protein with anti-synaptotagmin MAb. The arrow indicates synaptotagmin. M_r standards in kDa.

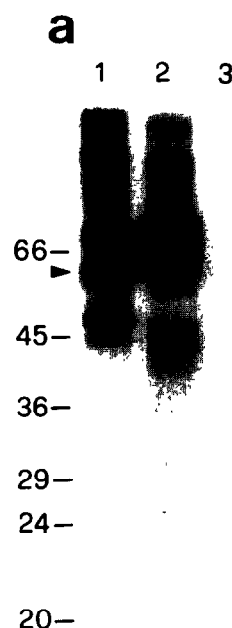


Fig. 2. (a) Immunoprecipitation of synaptotagmin from 250 μ g of vesicle proteins phosphorylated for 1 min in buffer A with 0.2 mM CaCl_2 /20 μ g/ml calmodulin. Antibodies used were: 1, synaptotagmin-MAb; 2, synapsin I-antiserum; 3, OKT8 MAb. The arrow indicates synaptotagmin. The phosphoprotein band at ~130 kDa in (1) corresponds to the synaptotagmin dimer, often observed in SDS electrophoresis of vesicle proteins.

prominent of these phosphoproteins was synapsin I (84–80K), which is a major substrate for CaMK II in synaptic vesicles [14]. It is well established that in vesicles synapsin I is endogenously phosphorylated on two sites upon the addition of Ca^{2+} and calmodulin. CaMK II is bound to the vesicle surface, where it has been found associated with synapsin [15]. In addition to synapsin and to the autophosphorylated CaMK II α -subunit (48K) a third, less prominent, phosphoprotein band became visible upon calmodulin addition, with an apparent M_r of 60K (Fig. 1). This band has an electrophoretic mobility close to that of the CaMK II β/β' -subunits (58–60K, less abundant than the α -subunit in forebrain). To investigate whether this phosphoprotein band contained synaptotagmin, phosphorylated vesicle proteins were immunoblotted with synaptotagmin MAb. As shown in Fig. 1, synaptotagmin comigrates with the labeled band. Therefore the protein was immunoprecipitated from phosphorylated vesicles. Fig. 2a shows that synaptotagmin-MAb immunoprecipitated a 60K phosphoprotein. Amounts of synapsin I and of CaMK II α -subunit were coimmunoprecipitated with synaptotagmin. The latter two proteins were not visible on the SDS gel of the immunoprecipitate (not shown), where, on the contrary, a synaptotagmin band was

clearly visible. This result was consistently obtained in five different immunoprecipitations, using different vesicle preparations. Immunoprecipitation of the same samples with antiserum against synapsin I produced a quite different pattern, with an evident heavy phosphosynapsin band (Fig. 2a).

Synaptotagmin contains a hypersensitive site of proteolysis, localized to the cytoplasmic domain, flanking the transmembrane region [16]. When vesicles are incubated with low concentrations of trypsin, the whole cytoplasmic portion (39K) of the protein becomes detached from the vesicle membrane [16]. As the only consensus site for phosphorylation by CaMK II is located at the C terminus in the cytoplasmic portion (R-H-W-S391), tryptic cleavage of this domain from phosphorylated vesicles should result in the formation of a phospho-cytoplasmic portion. Therefore phosphorylated vesicles were mildly trypsinized and tryptic supernatants were immunoprecipitated as described. As shown in Fig. 2b such a procedure led to the production of a phospho-cytoplasmic portion of synaptotagmin with the expected M_r . The cytoplasmic portion was still stained by the synaptotagmin-MAb (not shown).

4. DISCUSSION

The pattern of synaptic vesicle protein endogenous

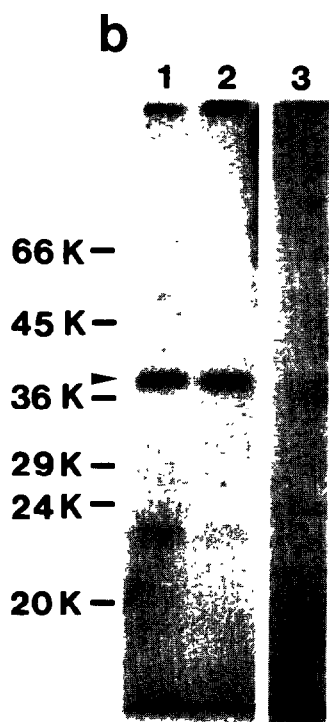


Fig. 2 (b) Immunoprecipitation of synaptotagmin cytoplasmic portion (39 kDa) from the vesicle tryptic supernatant. Phosphorylated vesicles (100 μ g) were incubated with 7 ng trypsin/ μ g of SV protein (lanes 1 and 3) or 27 ng trypsin/ μ g of SV protein (lane 2). Lanes 1–2, immunoprecipitation with synaptotagmin MAb; lane 3, immunoprecipitation with OKT8 MAb.

phosphorylation in the presence of Ca^{2+} /calmodulin was investigated. Under these conditions, as found by other authors [15], two major proteins become phosphorylated: synapsin I and the CaMK II α -subunit. In addition to these more prominent phosphoproteins, a third one, synaptotagmin, resulted to be phosphorylated only in the presence of Ca^{2+} /calmodulin. These data, and the fact that CaMK II is associated with purified SV [15], clearly suggest that synaptotagmin is phosphorylated by CaMK II itself. In previous experiments phosphorylation of synaptotagmin in intact SV may have been overlooked, because its electrophoretic mobility is close to that of CaMK II β/β' -subunits, and it is difficult to resolve the two proteins on SDS gels. However, immunoprecipitation of synaptotagmin, and of its cytoplasmic portion, shows that this vesicle protein is indeed endogenously phosphorylated. On the contrary, no major bands corresponding to CaMK II β/β' -subunits were found under the conditions used here, possibly because they overlap with phospho-synaptotagmin on SDS gels. Two possible explanations are suggested for the visualization of synapsin and the CaMK II α -subunit in the immunoprecipitate gels. A most evident explanation is that they are much more heavily labeled than synaptotagmin. Although neither of the two former proteins is visible on the gels, a faint trace is enough to appear in the autoradiography. The fact that synapsin and the CaMK II α -subunit form a complex on the vesicle membrane [15] may increase the possibility that a little amount of the complex remains trapped in the immunoprecipitate. A second explanation, which could be investigated, is that synaptotagmin too is associated to synapsin and CaMK II.

CaMK II has a broad substrate specificity, and has been implicated in several Ca^{2+} -regulated cell functions, including neurotransmitter release [17]. It would be interesting to investigate whether synaptotagmin undergoes a cycle of phospho-dephosphorylation in the vesicles. Phosphorylation of synaptotagmin by CaMK II could be a priming event (as it was shown for synapsin I), regulating its function in relation to the exo-endocytotic cycle of synaptic vesicles.

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